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Genetic differentiation of six sympatric species of *Isotomurus* (Collembola, Isotomidae); is there any difference in their microhabitat preference?

Antonio Carapelli*, Francesco Frati, Pietro Paolo Fanciulli and Romano Dallai

Department of Evolutionary Biology, University of Siena, via P.A. Mattioli 4, I-53100 Siena, Italy.

** Corresponding author.*

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Abstract

Genetic markers (allozymes and DNA sequences) were used to evaluate the taxonomic importance of small features of the pigmentation patterns among individuals of the genus *Isotomurus*. Such markers allowed the detection of six genetically well differentiated species, all coexisting in the same locality. The presence of loci fixed for alternative alleles ruled out the possibility of hybrid formation and confirmed that the taxa deserve the rank of species. In spite of the difficulty, at least in some cases, of recognizing the species on a morphological basis, they were all genetically well differentiated.

Genetic markers were also used to infer phylogenetic relationships. Reconstructions based on different data sets and different methods were not always concordant, but the close affinity of *Isotomurus palustris*, *I. ghibellinus* and *I. indipendente* can reasonably be accepted.

No apparent ecological difference was detected among the six species, however they showed substantial microgeographic segregation, with each species exhibiting preference for certain microhabitats. In the absence of a clear analysis of ecological differentiation, partial microhabitat preference is taken as a proof of such a differentiation. Microhabitat preference could be the cause and/or the effect of the speciation process.

A synthetic description of three new species (*Isotomurus ghibellinus* n. sp., *I. italicus* n. sp. and *I. indipendente* n. sp.), and of *I. unifasciatus* (erected to the rank of species) is given.

Keywords: *Isotomurus*, allozymes, ribosomal DNA, Polymerase Chain Reaction, microgeographic segregation, speciation.

Différenciation génétique de six espèces sympatriques du genre Isotomurus *(Collembola, Isotomidae); cette différenciation est-elle liée aux biotopes ?*

Résumé

Le système gène-enzyme et la séquence d'ADN ont été utilisés pour établir l'importance des détails morphologiques ainsi que de la pigmentation dans la systématique du genre *Isotomurus*. Les systèmes enzymatiques ont permis de mettre en évidence l'existence de six espèces bien caracterisées du point de vue génétique et vivant dans le même endroit. La présence de loci fixés pour différents allèles interdit la possibilité de formation d'hybrides et confère aux taxons le statut d'espèces. La méthode donne des résultats significatifs dans la diagnose des espèces lorsqu'elles ne sont pas bien différenciées morphologiquement.

Les marqueurs génétiques ont été également utilisés pour évaluer l'affinité phylogénétique des différentes espèces. Les reconstructions obtenues utilisant différentes méthodes ne conduisent pas toujours aux mêmes résultats, mais elles suggèrent toutefois une affinité très forte entre *Isotomurus palustris*, *I. ghibellinus* n. sp. et *I. indipendente* n. sp.

Bien que des différences écologiques bien définies n'aient pas été mises en évidence, chaque espèce étudiée montre une spécialisation microgéographique et une préférence pour un biotope particulier. Cette préférence pourrait être à l'origine (et/ou l'effet) du processus de spéciation.

Une description synthétique de trois nouvelles espèces (*Isotomurus ghibellinus* n. sp., *I. italicus* n. sp. et *I. indipendente* n. sp.), et de *I. unifasciatus* (élevé au niveau de bonne espèce) est donnée.

Mots-clés : Alloenzymes, *Isotomurus*, ADN ribosomique, réactions en chaîne de la polymérase, ségrégation microgéographique, spéciation.

INTRODUCTION

The classical interpretation of interspecific competition implies that when two or more species share the same ecological niche, coexistence is not possible and a dominant species will eliminate all competitors. This principle, known as competitive exclusion (Hardin, 1960) which subsequently evolved into the term of limiting similarity (MacArthur & Levins, 1967), has been generally accepted for a long time. Recently, however, it has been questioned, mostly on the basis of mathematical models (Abrams, 1983).

Various environmental parameters, such as temperature, humidity, soil chemical and physical structure (for edaphic species), type of vegetation, interactions with predators and number of competitors, are critical factors for species development and distribution, but availability and abundance of food are presumably the most important. Interspecific interactions regulate the equilibrium between two or more species and competition for food, in particular, may play a major role in the success of one species over another, especially when closely related organisms live in the same place. In *Drosophila*, it seems that the coexistence of related species using the same resources varies in space and time (Sevenster & van Alphen, 1993). Differences in life history strategies may therefore promote this coexistence (Chesson, 1985). For two congeneric species to live in the same site, niche differentiation is necessary, niche being interpreted in the broadest sense (*i.e.* to include all the previously mentioned environmental parameters in addition to life-cycle and life-style features).

According to the biological species concept (Mayr, 1942), two species are different if they show reproductive isolation. The detection of loci fixed for alternative allelic patterns in sympatric specimens is taken as the demonstration of the presence of reproductive isolation. In this sense, genetic markers, such as allozymes and DNA sequences, are a powerful tool for discriminating different species (Buth, 1984; Avise *et al.*, 1987) and inferring phylogenetic relationships (Avise, 1975; Simon *et al.*, 1994). They enable cryptic species to be detected (Duellman & Hillis, 1987) when species boundaries are difficult to define on a morphological basis (Hillis, 1987). In Collembola, this problem is particularly evident and many genera deserve careful analysis to verify the genetic uniformity of morphologically defined species. The genus *Isotomurus* is apparently very complicated in this respect (Cassagnau, 1987), with many varieties or subspecies erected on the basis of variable morphological characters. The recent introduction of additional morphological features (Deharveng & Lek, 1993) provides a deeper insight into intragenus taxonomy, but the genetic approach has been found to be extremely effective in the diagnosis of cryptic species (Carapelli *et al.*, 1995).

Very often, two or more congeneric species can be found together in the same site. In our experience, which includes examples from *Bilobella* (Dallai *et al.*, 1986), *Orchesella* (Frati *et al.*, 1992, 1994), *Allacma* (Fanciulli *et al.*, 1994) and *Isotomurus* (Carapelli *et al.*, 1995), species of these genera have always been well differentiated by diagnostic enzyme loci, but it has never been evident whether different ecological specializations prevent direct competition between them. In some cases, there is slight microgeographic segregation, in the sense that certain species are more abundant in one particular site than another, but many sites of overlap are always present. Sometimes competition seems to be avoided on the basis of food specialization (Fjellberg, 1985), but microhabitat preference has also been demonstrated between sympatric congeneric species of *Vertagopus* (Leinaas & Fjellberg, 1985).

Allozymes are another way to study the genetic structure of species. Estimates of genetic diversity can be correlated with several ecological and historical parameters (Nevo *et al.*, 1984).

The aim of this study was to evaluate the degree of genetic differentiation between six presumed species of *Isotomurus* found in the same sampling site at Radi near Siena, Central Italy. There, we collected specimens of *Isotomurus* along a small stream for about two years. The specimens had different, but variable, pigmentation patterns. Genetic markers were used to evaluate the importance of these differences in the diagnosis of species; the independent data sets they provided were used to construct phylogenetic trees illustrating the evolutionary relationships between taxa.

MATERIALS AND METHODS

Collecting site

The collecting site was at Radi, near Siena, in the area of the Sorra, a small stream which runs between a wood and cultivated fields. *Figure* 1 is a diagram of the collecting site in transverse section. The area was about 10 meters wide and included a 30-meters strip along the length of the stream. Five microhabitats, indicated

with letters from *a* to *e*, were identified on the basis of the physical structure of the soil and the vegetation. The wood consisted of trees of *Quercus pubescens* and *Populus nigra* (microhabitat *a*). *Isotomurus* were found in the litter layer of this area. The wood was separated from the stream by a small path which ran parallel to the stream at a higher level (microhabitat *b*). This was characterized by fallen leaves, various types of shrubs (*Cornus sanguinea, Cytisus sessilifolius, Ligustrum vulgare, Acer campestre, Crataegus monogyna, Pyracantha coccinea*), mosses and grasses of several species (*Centaurea* sp., *Dactylis* cf. *hispanica, Carex flacca, Inula salicina* and *Plantago* sp.) and young specimens of *Rosa* cf. *canina*. Specimens of *Isotomurus* were found along the path in the site of puddles. A small slope running down to the stream (microhabitat *c*) was mainly characterized by the above shrubs and small trees (*Quercus cerris* and *Q. ilex*). The banks of the stream consisted of a steep wall of clay with no vegetation (microhabitat *d*) and stones (microhabitat *e*). Microhabitat *d* was always soaked with water and shaded by a dense overhead canopy. Microhabitat *e* was occasionally flooded but was also more exposed to sunlight and usually dried quite quickly. On this side of the stream, agriculture may have reduced the original living space for *Isotomurus* species.

Sample analysis

Specimens of *Isotomurus* spp. were collected with mechanical aspirators over a two-year period (1993-1994). Systematic sampling was performed during favorable seasons (September-June) and care was taken to sample homogeneously in the five microhabitats. In the summer (July-August) the number of specimens decreased noticeably, and increased again a few days after heavy rain. The live specimens were taken to the laboratory, sorted on the basis of their pigmentation patterns, killed with liquid nitrogen and stored at $-80°C$.

It was easy to recognize *I. maculatus* (Carapelli *et al.*, 1995; *fig.* 2a) and *I. palustris* (*fig.* 2d). Another group of individuals was easily distinguishable from the rest because their pattern of pigmentation was identical to *I. balteatus* (*fig.* 2b). A fourth group of specimens was also distinguishable because their pigmentation was characterized by uniform brownish

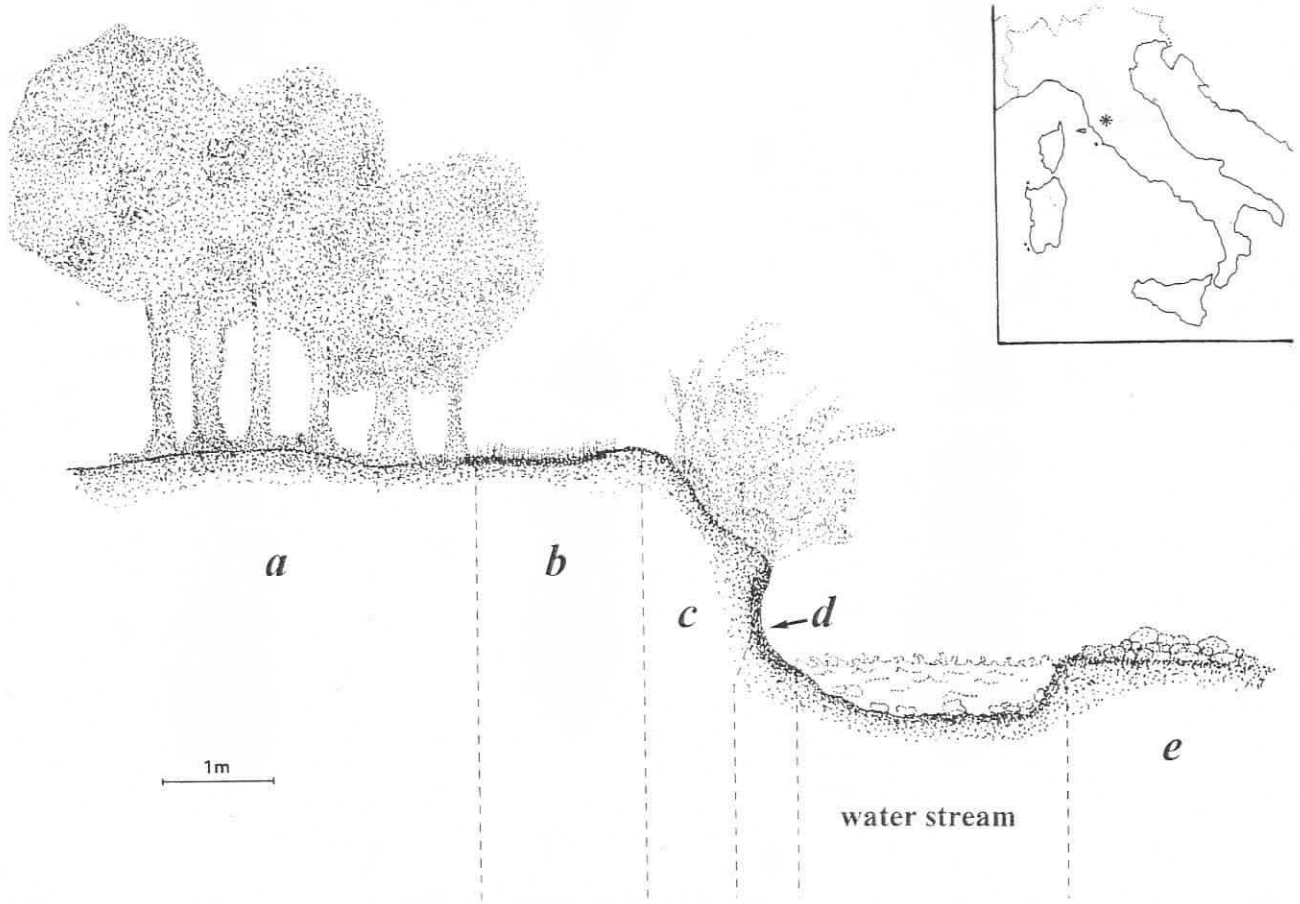

Figure 1. – Diagram in transverse section of sampling site showing five different microhabitats (*a* to *e*). See text for vegetation of each microhabitat. Geographic position of sampling site shown in the box (*).

coloring without any band, stripe or patch of pigment (*fig.* 2c). Among the remaining specimens, patterns of colour were very heterogeneous, generally resembling those of *I. palustris* (*sensu* Poinsot-Balaguer, 1972). Only after detecting diagnostic enzyme loci (*see* later), which were congruent with features of the pigmentation, was it possible to recognize the small differences in the pigmentation which differentiated two potentially distinct species from *I. palustris* (*figs.* 2e and 2f). In the light of this uncertainty, we decided to use allozymes and DNA sequences to test whether six species were actually present in the same sampling site.

Allozyme analysis was performed by starch gel electrophoresis according to the technical and staining procedures described by Murphy *et al.* (1990). Twelve loci yielded interpretable banding patterns: arginine kinase (*Ark-1-2*: Enzyme Com-

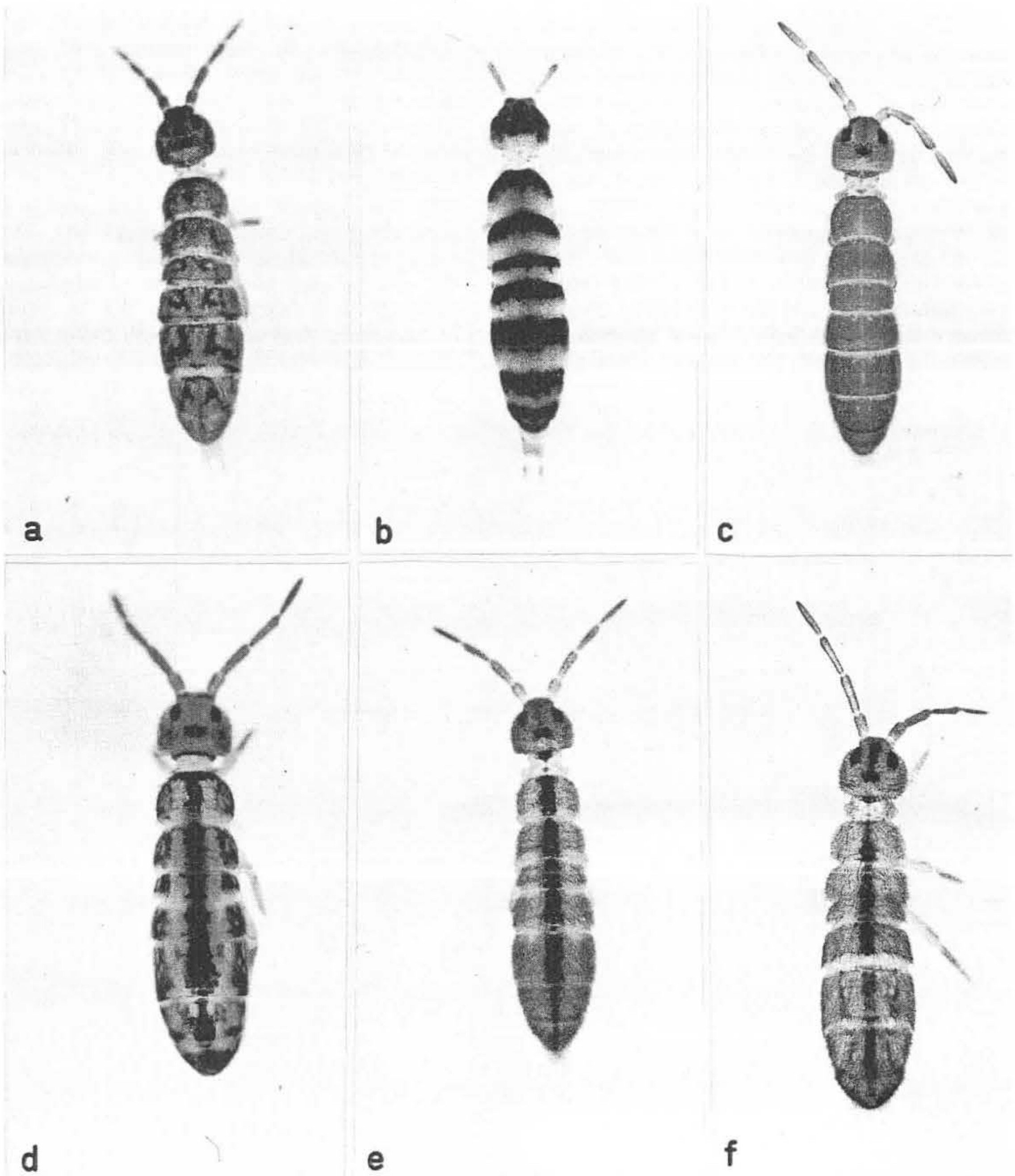

Figure 2. – Photographs of the six species of *Isotomurus*: a) *I. maculatus* (18×); b) *I. ghibellinus* (18×); c) *I. italicus* (37×); d) *I. palustris* (23×); e) *I. unifasciatus* (26×); f) *I. indipendente* (26×). The first three species are quite easily distinguishable, but the last three are much more similar, and also more variable.

mission number 2.7.3.3), glyceraldehyde-3-phosphate dehydrogenase (*G3pdh*: EC 1.2.1.12), hexokinase (*Hk*: EC 2.7.1.1), isocitrate dehydrogenase (*Idh-1-2*: EC 1.1.1.42), malate dehydrogenase (*Mdh-1-3*: EC 1.1.1.37), mannose-6-phosphate isomerase (*Mpi*: EC 5.3.1.8), phosphoglucomutase (*Pgm*: EC 5.4.2.2), phosphohexose isomerase (*Phi*: EC 5.3.1.9.) and pyruvate kinase (*Pk*: EC 2.7.1.40). Genotype frequencies were processed by the computer program BIOSYS-1 (Swofford and Selander, 1981) which was used to calculate alleles frequencies at each locus (*table* 1) and several parameters of intra- and interspecific genetic diversity (see *tables* 2 and 3). When a population is completely panmictic and no selection is acting upon allele frequencies, the percentage of heterozygous individuals is directly correlated with initial allele frequencies (Hardy-Weinberg equilibrium). If the observed amount of heterozygosity differs from the expected value, it may be supposed that some external factor is involved in decreasing or increasing the number of heterozygous individuals. A distance-based phylogenetic analysis was performed by constructing UPGMA (Unweighted Pair Group Method with Arithmetic mean) and Wagner dendrograms (using BIOSYS-1), and a Neighbor-joining tree (NJ: Saitou and Nei, 1987: *fig.* 3) using the program MEGA (Kumar *et al.*, 1993). Allele frequencies were also processed by the computer program FREQPARS (Swofford and Berlocher, 1987) which yielded a Maximum Parsimony (MP) phylogenetic tree directly based on observed allele frequencies (*fig.* 4).

Total DNA was extracted from one specimen of each presumed species according to the procedure of Simon *et al.* (1991) with slight modifications to purify nuclear DNA as well. A fragment of the small nuclear ribosomal DNA subunit (276 bp.) was amplified by the Polymerase Chain Reaction (PCR; Mullis *et al.*, 1986). A single pair of primers was used for PCR and sequencing: D3a (5′-GACCCGTCTTGAAACACGGA-3′) and D3b (5′-TCGGAAGGAACCAGCTACTA-3′). PCR was performed for 36 cycles in 100 μl reactions with the following profile: denaturation at 95° for 1 min., annealing at 50° for 1 min. and extension at 72° for 1 min. and 10 sec. Total amplification products were run on a 1% low melting point agarose gel, the band excised from the gel and the DNA extracted by phenol/phenol-chloroform/chloroform extraction followed by ethanol precipitation. DNA was then directly sequenced according to the double-strand method of Hsiao (1993). Sequences were aligned by eye using the computer program ESEE (Cabot & Beckenbach, 1989), while MEGA was used to construct a distance-based neighbor-joining tree (*fig.* 5) and a MP phylogenetic tree.

The nucleotide sequences reported in this paper have been deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession numbers X84951-X84957.

RESULTS

Allozymes

As shown in *table* 1, none of the twelve loci scored had the same allele(s) in the six presumed species. Five of them (*Ark-2, G3pdh, Hk, Mdh-3* and *Pk*) were fixed in all six taxa. The other seven loci showed variable degrees of polymorphism, the most polymorphic being the *Pgm* locus (seven alleles). The distribution of allele frequencies demonstrated the presence of six different species, the reproductive isolation of which is testified by the loci fixed for alternative alleles. The name *Isotomurus ghibellinus* was given to the species resembling *I. balteatus* (*fig.* 2b) while *Isotomurus italicus* was assigned to the species with the uniform brownish colouration (*fig.* 2c). For the two species similar to *I. palustris*, the names *Isotomurus unifasciatus* (corresponding to *I. palustris* f. *unifasciata* [Börner, 1901]: *fig.* 2e) and *Isotomurus indipendente* (*fig.* 2f) were chosen.

The species were all genetically quite variable, with 33% to 50% of polymorphic loci, and more than a mean of 1.6 alleles per locus (*table* 2). Curiously, the mean observed heterozygosity was always considerably lower than expected under Hardy-Weinberg equilibrium. Furthermore, the genetic distance values were quite high which confirms clear differentiation of the six species. The most distant species appeared to be *I. unifasciatus* and *I. indipendente* (D = 1.992), and the most similar were the pairs *I. palustris – I. ghibellinus* (D = 0.350) and *I. palustris – I. indipendente* (D = 0.412). Phylogenetic reconstructions gave slightly different results. Two trees are shown: a NJ tree based on Nei's (1978) genetic distance (*fig.* 3) and a tree constructed by FREQPARS (*fig.* 4). They are quite similar, always clustering *I. palustris, I. indipendente* and *I. ghibellinus* together and showing *I. italicus* as the most divergent species. The cluster *I. palustris, I. indipendente* and *I. ghibellinus* seems to be quite solid, as it was also present in the UPGMA and the Wagner trees (not shown). The two trees differed with regard to the position of *I. maculatus*.

DNA

The 276 bp fragment of the six species was easily aligned and no gaps or insertions were present. 32 positions were variable and they were concentrated at the 5′-end, with only 5 variable sites in the last 161 bases of the fragment. The percentage of interspecific sequence divergence was quite low, showing that this fragment is relatively well conserved in the genus *Isotomurus*. The NJ tree shown in *fig.* 5 is somewhat different from those based on allozyme data, the only congruency being the affinity between *I. palustris* and *I. ghibellinus*. The sequence of the Symphypleonan collembolan *Allacma fusca* was used

Table 1. – Allele frequencies observed at the 12 loci scored in the six taxa. (N): sample size*.

	Population					
Locus	*I. maculatus*	*I. palustris*	*I. unifasciatus*	*I. indipendente*	*I. ghibellinus*	*I. italicus*
Ark-1						
(N)	44	55	23	68	64	25
A	1.000	0.000	0.000	0.000	0.000	0.000
B	0.000	0.036	0.000	0.000	0.102	1.000
C	0.000	0.964	0.000	0.000	0.898	0.000
D	0.000	0.000	0.000	1.000	0.000	0.000
E	0.000	0.000	1.000	0.000	0.000	0.000
Ark-2						
(N)	83	58	84	53	74	56
A	1.000	0.000	1.000	0.000	1.000	0.000
B	0.000	1.000	0.000	1.000	0.000	1.000
G3 pdh						
(N)	32	74	26	31	63	15
A	1.000	1.000	0.000	1.000	1.000	0.000
B	0.000	0.000	1.000	0.000	0.000	1.000
Hk						
(N)	43	33	23	27	43	31
A	0.000	0.000	1.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	1.000
C	1.000	0.000	0.000	0.000	0.000	0.000
D	0.000	0.000	0.000	0.000	1.000	0.000
E	0.000	1.000	0.000	1.000	0.000	0.000
Idh-1						
(N)	40	47	21	23	87	10
A	1.000	0.000	1.000	0.000	0.000	0.000
B	0.000	0.000	0.000	1.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.000	0.250
D	0.000	0.000	0.000	0.000	0.000	0.750
E	0.000	1.000	0.000	0.000	1.000	0.000
Idh-2						
(N)	37	27	16	20	83	8
A	0.527	0.685	0.625	0.025	0.651	0.875
B	0.000	0.000	0.344	0.000	0.000	0.125
C	0.473	0.315	0.031	0.975	0.349	0.000
Mdh-1						
(N)	40	103	39	40	46	25
A	1.000	0.806	1.000	0.000	1.000	0.000
B	0.000	0.194	0.000	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.000	1.000
D	0.000	0.000	0.000	1.000	0.000	0.000
Mdh-3						
(N)	59	133	50	80	159	42
A	1.000	0.000	1.000	0.000	0.000	1.000
B	0.000	1.000	0.000	1.000	1.000	0.000
Mpi						
(N)	51	28	15	11	31	5
A	0.412	0.482	0.667	0.455	0.177	0.000
B	0.020	0.000	0.000	0.000	0.000	0.000
C	0.059	0.464	0.100	0.091	0.790	1.000
D	0.510	0.054	0.233	0.455	0.032	0.000

Table 1. (continued).

Locus	*I. maculatus*	*I. palustris*	*I. unifasciatus*	*I. indipendente*	*I. ghibellinus*	*I. italicus*
Pgm						
(N)	*36*	*22*	*12*	*13*	*29*	*18*
A	0.389	0.977	0.500	0.577	0.034	0.389
B	0.042	0.000	0.000	0.077	0.000	0.000
C	0.181	0.000	0.042	0.077	0.000	0.056
D	0.361	0.000	0.125	0.192	0.966	0.333
E	0.000	0.000	0.000	0.000	0.000	0.111
F	0.028	0.023	0.208	0.077	0.000	0.083
G	0.000	0.000	0.125	0.000	0.000	0.028
Phi						
(N)	*342*	*30*	*26*	*32*	*62*	*35*
A	0.603	0.817	0.750	0.719	0.653	0.000
B	0.397	0.183	0.173	0.172	0.226	0.129
C	0.000	0.000	0.000	0.000	0.000	0.743
D	0.000	0.000	0.077	0.109	0.121	0.129
Pk						
(N)	*12*	*12*	*19*	*15*	*36*	*10*
A	1.000	1.000	0.000	1.000	1.000	1.000
B	0.000	0.000	1.000	0.000	0.000	0.000

(Population spans all species columns.)

* Sample size discrepancies within and between populations and loci are due to: a) the need to screen more individuals when levels of polymorphism are high; and b) the coupling of two different enzyme stains in the same electrophoretic run, their choice depending on migration rate and suitable electrophoretic buffers.

as outgroup reference. Because of the small number of variable sites, the MP tree was almost totally unresolved (not shown) and, for the same reason, DNA data was, in this case, less powerful than allozymes for revealing phylogenetic relationships.

Microgeographic segregation

Collection of specimens of *Isotomurus* in all seasons of the year allowed us to evaluate their preferences for the different microhabitats. As shown in *fig.* 6, each species exhibited a preference for one of the five microhabitats. None of them was exclusively related to a single microhabitat but they could all occasionally be found everywhere. *I. indipendente* and *I. ghibellinus,* however, seemed to be nearly always restricted to microhabitats *b* and *d*, respectively, and only rarely found elsewhere. *I. palustris* and *I. unifasciatus* seemed to have a wider range of activity. The first is most frequent in microhabitat *a*, but it is also constantly found in microhabitats *c,*

Table 2. – Estimates of genetic variability in the six species. Standard errors are in brackets. ss: sample size; A: mean number of alleles per locus; P: percentage of polymorphic loci (under the 0.99 criterium); Ho: observed mean heterozygosity; He: expected mean heterozygosity under Hardy-Weinberg equilibrium (unbiased estimate [see Nei, 1978]).

Population	ss	A	P	Mean heterozygosity Ho	He
1. *I. maculatus*	42.6 (4.9)	1.8 (0.4)	33.3	0.142 (0.063)	0.188 (0.081)
2. *I. palustris*	51.8 (10.4)	1.6 (0.2)	50.0	0.119 (0.048)	0.144 (0.059)
3. *I. unifasciatus*	29.5 (5.8)	1.8 (0.4)	33.3	0.128 (0.058)	0.177 (0.078)
4. *I. indipendente*	34.4 (6.4)	1.8 (0.4)	33.3	0.084 (0.045)	0.145 (0.074)
5. *I. ghibellinus*	64.8 (10.3)	1.6 (0.2)	41.7	0.093 (0.042)	0.131 (0.057)
6. *I. italicus*	23.3 (4.5)	1.8 (0.4)	33.3	0.104 (0.058)	0.149 (0.071)

Table 3. – Estimates of genetic distance (above the diagonal: D [Nei, 1978]) and genetic similarity (below the diagonal: S [Rogers, 1972]).

Population	*I. maculatus*	*I. palustris*	*I. unifasciatus*	*I. independente*	*I. ghibellinus*	*I. italicus*
1. *I. maculatus*	***	0.795	0.575	0.990	0.608	1.251
2. *I. palustris*	0.459	***	1.293	0.412	0.350	1.072
3. *I. unifasciatus*	0.573	0.311	***	1.992	1.124	1.215
4. *I. indipendente*	0.421	0.635	0.230	***	0.889	1.431
5. *I. ghibellinus*	0.552	0.691	0.360	0.423	***	1.290
6. *I. italicus*	0.335	0.346	0.341	0.289	0.289	***

d and *e*; the second prefers microhabitat *b* but also occurs frequently in microhabitats *a*, *c* and *e*.

Description of species

In the light of the genetic evidence and the correspondence between genetic markers and pigmentation patterns, three new species are described, and the rank of species for *I. palustris* f. *unifasciata* (Börner, 1901) was determined. From a morphological point of view, only a few characters have been studied, but none of them showed interspecific differences. In particular, all of the species had 3+3+1 trichobothria on Abd. II, III and IV, and an identical arrangement of setae S on Abd. V. For these characters, they have to be included in the *Isotomurus palustris* group (*sensu* Deharveng & Lek, 1993), to which *I. maculatus* and *I. palustris* also belong. A more accurate morphological study is underway but in the following description, only pigmentation patterns are taken into account. For the moment, in fact, this character is the only one, apart from genetic markers, that distinguishes the species.

Isotomurus ghibellinus n.sp. (*fig.* 2b)

Body length: 2-3 mm. Each tergite has a horizontal band of dark pigment in the anterior part, but is devoid of pigment in the posterior part. Background colour is yellowish or whitish. Inter-individual variability is observed in the thickness of the dark band on each tergite.

Material: Radi (Siena province); holotype and 5 paratypes preserved in the R. Dallai collection. Other collecting localities are: Bocca Serriola (Perugia province), Alpi Apuane (Massa Carrara province).

Isotomurus italicus n.sp. (*fig.* 2c)

Body length: 1-2 mm. Background colour brownish or yellowish. No band, stripe or particular pigment mark is present.

Material: Radi (Siena province); holotype and 5 paratypes preserved in the R. Dallai collection. Other collecting localities are: Circeo (Latina province), Gerfalco and Manciano (Grosseto province), Giglio island.

Isotomurus unifasciatus (Börner, 1901) (*fig.* 2e)

Syn: *Isotoma palustris* var. *unifasciata* (Börner, 1901)

Isotomurus palustris f. *unifasciata* (Stach, 1947)

Body length: 2-3 mm. Background colour whitish, yellowish, or greenish. Dorsum with a continuous black or violet longitudinal stripe from Thor. II to Abd. VI, rarely fainter on the last abdominal tergites. Symmetrical pigmentation spots sometimes present laterally on Abd. VI.

Material: Collecting localities are: Radi (Siena province), Gerfalco (Grosseto province), Bocca Serriola (Perugia province), Pennapiedimonte (Chieti province), Gran Sasso (L'Aquila province), Montségur and Fontextorbs (Ariege-FR).

Remarks: We have not had the possibility to study topotypic specimens of *I. palustris* f. *unifasciata* (Börner, 1901), but the pigmentation pattern of the specimens from Radi corresponds exactly to the original description by Börner (1901) and Stach (1947). In addition, this species is quite uniform, since specimens collected from South France to South Italy all show the same pattern of pigmentation and are genetically very homogeneous (unpublished study). For these reasons, we believe that our specimens correspond to *I. palustris* f. *unifasciata* (Börner, 1901), which must, therefore, be erected to the rank of species. Further analysis, both morphological and genetic, on material from the topotypic sampling site will be attempted in the future.

Isotomurus indipendente n.sp. (*fig.* 2f)

Body length: 2-3 mm. Background colour yellowish, greenish or brownish. Tergites with a longitudinal stripe running from Thor. II to Abd. VI, very often interrupted on Abd. IV. Faint markings on each side of abdominal tergites, and pigmentation spots on the lateral side of Abd. VI.

Material: Radi (Siena province); holotype and 5 paratypes preserved in R. Dallai collection. Other collecting localities are: Gerfalco (Grosseto province), Fortore (Foggia province), Bocca Serriola (Perugia province), Salin de Badon, Fos sur Mer, Pioch Badet (Camargue-FR).

DISCUSSION

The present results are a further example of the utility of genetic markers in taxonomic questions. Genetic data clearly demonstrated that the six presumed species are in fact well differentiated and deserve the status of species. The presence of interspecific reproductive isolation is testified by the lack of hybrids in the loci fixed for alternative alleles. Several loci, like *Ark-1-2, G3pdh, Hk, Idh-1, Mdh-1-3* and *Pk*, can be used to discriminate certain species from others (*table* 1). In this case, genetic markers support the taxonomic value of the small differences in pigmentation patterns which, at first glance, could be mistakenly regarded as mere intraspecific variation. The genetic approach is therefore particularly useful in species-rich genera, such as *Isotomurus*, in which characters for species diagnosis are difficult to find and may pass for intraspecific variation (Cassagnau, 1987; Deharveng & Lek, 1993).

Although we found a nice correlation between small differences in pigmentation patterns and the presence of loci fixed for alternative alleles, this correlation unfortunately does not hold for all species of the genus. In some examples, in fact, pigmentation patterns were identical, but the species were clearly differentiated for other characters. This is the case for *I. ghibellinus* which has exactly the same coloring as *I. balteatus* (*sensu* Poinsot-Balaguer & Ferard, 1983), but the number of abdominal trichobothria demonstrates that they are different species.

Estimates of intraspecific variability (*table* 2) are quite high for all six species, but this data cannot be compared with other taxa because of the small number of loci scored and the dependence of these estimates on the number and choice of loci (Simon & Archie, 1985). It is worth noting, however, that the most polymorphic species, *I. palustris* (P = 0.50), was also the species with the widest range of occurrence in the sampling site and it therefore seems to have a higher capacity to adapt to a heterogeneous environment. This higher capacity could be related to genetic variability (Ayala, 1968). The deficiency of heterozygotes is difficult to explain but it has been observed in other species with microgeographically structured subpopulations (Piertney & Carvalho, 1994) and may be related to small population size and reduced dispersal ability causing higher levels of inbreeding.

Despite of the initial difficulty of recognizing some of the species on a morphological basis, they all turned out to be quite well differentiated genetically. Some pairs of species had up to 67% (8/12) of loci fixed for alternative alleles (*I. unifasciatus – I. indipendente*). This pair also had the highest distance (D = 1.992). Interestingly, these two species shared the same preference for microhabitat *b* and this strict sympatry may have reinforced their genetic differentiation. Many other pairs of species had values of D > 1 (*table* 3); among them *I. palustris* and *I. italicus* were also abundant in the same microhabitat (*a*) and had 42% (5/12) of loci fixed for alternative alleles.

Allozyme and DNA data both provided evidence of clear differentiation of species. However, phylogenetic reconstructions using both data sets and different tree-building algorithms did not give concordant results, meaning that the phylogenetic signal in the data is fairly weak. In particular, the DNA data does not appear to be very informative for this purpose because of the small number of variable sites. Allozyme data are more solid and the cluster *I. palustris, I. ghibellinus* and *I. indipendente* emerges in most of the reconstructions (*figs.* 3 and 4). Two of the species (*I. palustris* and *I. indipendente*) in this cluster, which is also supported by other tree-building methods, are morphologically similar, whereas *I. ghibellinus* has a very peculiar pigmentation pattern (*fig.* 2). There does not seem to be a clear correlation between similarity of pigmentation patterns (in terms of which *I. palustris, I. unifasciatus* and *I. indipendente* are the most similar [*fig.* 2]) and the phylogenetic relationships depicted in *figs.* 3, 4 and 5. Preliminary sequence information on the mitochondrial COII gene (Frati *et al.*, 1995) supports the evidence that *I. maculatus* and *I. unifasciatus* are more closely related to each other than they are to *I. palustris.*

We were not able to find any ecological differentiation between the species which could explain their microgeographic segregation. However, this segregation is quite evident (*fig.* 6) and several hypotheses can be formulated on the basis of the characteristics of the different microhabitats. *I. ghibellinus* is mostly found in microhabitat *d* which is the wettest area of the site. Microhabitat *b* is also very moist and the insects (mostly *I. unifasciatus* and *I. indipendente*) seem to concentrate on the site of puddles which form there. Mimicry and escape from predators could also play a role in microhabitat

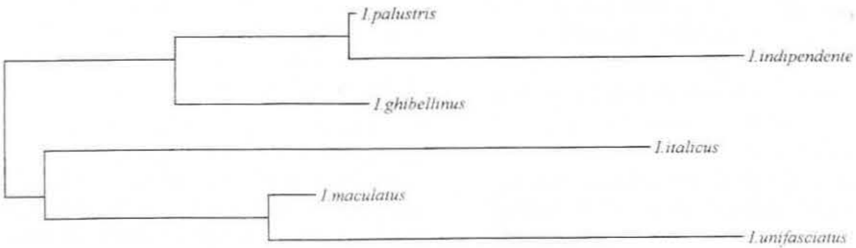

Figure 3. – Neighbor-joining tree based on Nei's (1978) genetic distance calculated from allozyme frequencies.

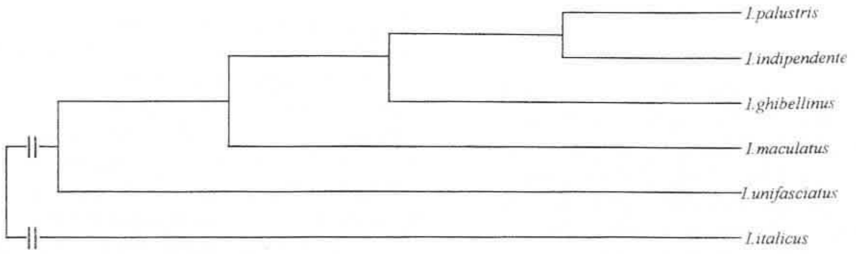

Figure 4. – Phylogenetic tree constructed using FREQPARS on the basis of untransformed allozyme frequencies.

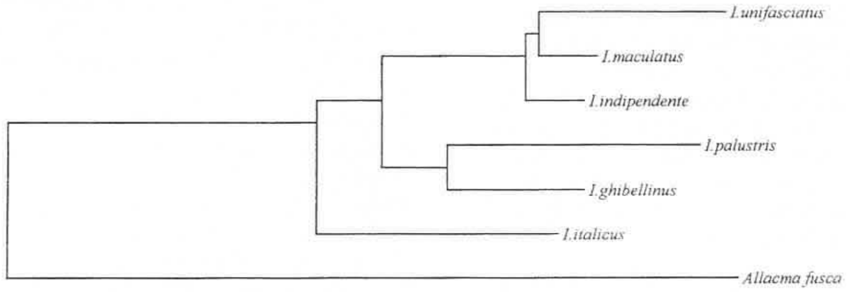

Figure 5. – Neighbor-joining tree based on Kimura 2-parameter distance calculated from the sequences of the D3 fragment.

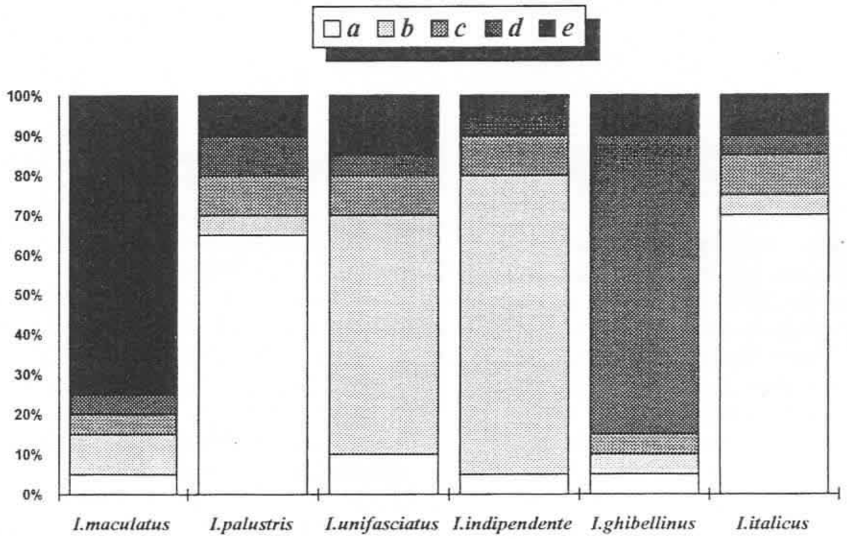

Figure 6. – Histogram showing microhabitat preferences of the six species.

choice. *I. unifasciatus* has a green background color and a clear preference for mosses and grasses in microhabitat *b*. *I. ghibellinus* is yellowish in color and could have an advantage from living in the clay of microhabitat *d*, where it is less visible than elsewhere. The adaptive value of coloring has long been known (Robinson, 1969). Several possible types of eucrypsis have been described in which patterns of pigmentation directly contribute to self-defense. The examples of *I. unifasciatus* and *I. ghibellinus* could be ascribed to homochromy (when the coloration resembles that of the background). Also countershading and obliterative coloration could explain some of the models of pigmentation observed in *Isotomurus* species. All these types of eucryptic coloration give the insects a powerful defense system, hiding them from potential predators. It is known (Bauer, 1971) that some predators hunt collembolan species by visual clues and mimicry could be an efficient defensive strategy for these species. No experimental data, however, are available on specific predation pressure upon *Isotomurus* species.

Environmental heterogeneity could itself be the cause of the occurrence of so many congeneric species in the same locality. The presence of spatially correlated but ecologically diversified microhabitats may also have been a suitable setting for the speciation process. Diversifying selection could have been the

guiding force for such a process and Radi can be regarded as a sort of model environment for this type of sympatric speciation. Whether or not competition still occurs, it could have been the cause of the establishment of microgeographic segregation between incipient species and this is more clearly evident in this site due to the diversification of the habitats in a reduced area.

These ecological motives, however, are not the only possible hypotheses to explain speciation. The Italian peninsula underwent radical geodynamic rearrangements during the Tertiary and Quaternary (Mantovani *et al.*, 1992 and references therein), with extensive land fragmentation. Geographic isolation might have been the initial step in a speciation process which was completed after sympatry was restored. It is currently impossible to decide which of these factors was the most important in the speciation process of the genus *Isotomurus* and indeed the synergic combination of several factors is also probable.

Concluding remarks on taxonomy

In the present study, genetic data provided a tool for demonstrating the status of species of various morphologically similar *Isotomurus* specimens. In the light of the biological species concept, genetic markers, and allozymes in particular, provide evidence for reproductive isolation among natural sympatric populations. Without the molecular evidence it would have been difficult to decide whether the small differences in pigmentation patterns (especially among *I. palustris*, *I. unifasciatus* and *I. indipendente*) were in fact an indication of reproductive isolation.

The characterization of *I. ghibellinus*, for instance, is a key problem. Specimens from Radi clearly do not belong to the same species as the French *I. balteatus* described by Poinsot-Balaguer and Ferard (1983) because of the different number of abdominal trichobothria (3+3+1 vs. 0+1+1 or 0+0+1). *I. balteatus* was originally described by Reuter (1876) from Scandinavian material, but his description is poor and no mention is made of the number of trichobothria. Accurate morphological observations suggest that *I. balteatus* described by Poinsot-Balaguer and Ferard (1983) is different from Scandinavian *I. balteatus* (as noted by Christiansen and Bellinger [1992: p. 154]) because of the absence of the typical mucronal seta. Many other species with the same model of pigmentation as *I. balteatus* have been described (see: Poinsot-Balaguer & Ferard, 1983). A preliminary screening of a few morphological characters shows that our specimens do not correspond to any of these species, which is further evidence that the specimens found in Radi (and other localities in Central Italy and in the Pyrenees) are a new taxon. The presence of the same particular model of pigmentation in different species from several parts of the world may be related to the cryptic value of the pigmentation and to its possible defensive role.

A similar problem exists for the determination of *I. palustris*. This species was originally described by Müller (1776) from Danish material, and revised by Poinsot-Balaguer (1972) on the basis of specimens from the Camargue (France). Preliminary genetic data (mitochondrial COII gene [Frati *et al.*, 1995]) show that specimens from the Camargue correspond to *Isotomurus indipendente* and not to *I. palustris*. On the other hand, the same genetic data suggests that other Italian populations of *I. palustris* have a sufficiently high genetic divergence to constitute new specific entities.

These two examples give an idea of the problems involved in describing species of the genus *Isotomurus*. We hope that careful study of morphological characters, supported by the biochemical and molecular evidence, will allow us to give a detailed description of these species.

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REFERENCES

Abrams P. (1983). – The theory of limiting similariry. *Ann. Rev. Ecol. Syst.*, **14**, 359-376.

Avise J. C. (1975). – Systematic value of electrophoretic data. *Syst. Zool.*, **23**, 465-481.

Avise J. C., Arnold J., Ball R. M., Bermingham E., Lamb T., Neigel J. E., Reeb C. A. & Saunders N. C. (1987). – Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Ann. Rev. Ecol., Syst.*, **18**, 269-292.

Ayala F. J. (1968). – Genotype, environment and population numbers. *Science*, **162**, 1453-1459.

Bauer T. (1971). – Prey capture and structure of the visual space of an insect that hunts by sight on the litter layer (*Notiophilus biguttatus* F., Carabidae, Coleoptera). *Behav. Ecol. Sociobiol.*, **8**, 91-97.

Börner C. (1901). – Zur Kenntnis der Apterygoten-Fauna von Bremen und der Nachbardistrickte. *Abh. d. Naturw. Ver. zu Bremen*, Bd. **XVII**, 1-141.

Buth D. G. (1984). – The application of electrophoretic data in systematic studies. *Ann. Rev. Ecol. Syst.*, **15**, 501-522.

Cabot E. L. & Beckenbach A. T. (1989). – Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. *Computer Applications in Biosciences*, **5**, 233-234.

Carapelli A., Fanciulli P. P., Frati F. & Dallai R. (1995). – The use of genetic markers for the diagnosis of sibling species in the genus *Isotomurus* (Insecta, Collembola). *Boll. Zool.*, **62**, 71-76.

Cassagnau P. (1987). – A propos des types de coloration chez *Isotomurus palustris* (Müller) (Collemboles). *Rev. Ecol. Biol. Sol*, **24**, 85-89.

Chesson P. L. (1985). – Coexistence of competitors in spatially and temporally varying environments: a look at the combined effects of different sorts of variability. *Theor. Popul. Biol.*, **28**, 263-287.

Christiansen K. A. & Bellinger P. F. (1992). – Insects of Hawaii. Vol. 15: Collembola. Honolulu: University of Hawaii Press, 445 p.

Dallai R., Fanciulli P. P. & Petrucci R. (1986). – Enzyme diversity in the genus *Bilobella* (Insecta, Collembola). *Rev. Ecol. Biol. Sol*, **23**, 333-348.

Deharveng L. & Lek S. (1993). – Remarques sur la morphologie et la taxonomie du genre *Isotomurus* Börner, 1903 et description de deux espèces nouvelles de France (Collembola: Isotomidae). *Ann. Soc. Entomol. Fr. (N.S.)*, **29**, 245-259.

Duellman W. E. & Hillis D. M. (1987). – Marsupial frogs (Anura: Hylidae: *Gastrotheca*) of the Ecuadorian Andes: resolution of taxonomic problems and phylogenetic relationships. *Herpetologica*, **43**, 135-167.

Fanciulli P. P., Frati F., Carapelli A. & Dallai R. (1994). – Livelli di variabilità e flusso genico in due specie del genere *Allacma* (Collembola: Sminthuridae). *Atti del XVII Congresso Nazionale italiano di Entomologia*, 73-76.

Fjellberg A. (1985). – Recent advances and future needs in the study of Collembola biology and systematics. *Quaestiones Entomologicae*, **21**, 559-570.

Frati F., Fanciulli P. P. & Dallai R. (1992). – Genetic diversity and taxonomy in soil-dwelling insects: the genus *Orchesella*. *J. Heredity*, **83**, 275-281.

Frati F., Fanciulli P. P. & Dallai R. (1994). – Further acquisitions on systematics relationships within the genus *Orchesella* (Collembola, Entomobryidae) using allozymes. *Acta Zoologica Fennica*, **195**, 35-43.

Frati F., Carapelli A., Fanciulli P. P. & Dallai R. (1995). – The genus *Isotomurus*: where molecular markers help to evaluate the importance of morphological characters for the diagnosis of species. *Polskie Pismo Entomol.*, **64**, in press.

Hardin G. (1960). – The competitive exclusion principle. *Science*, **131**, 1292-1297.

Hillis D. M. (1987). – Molecular versus morphological approaches to systematics. *Ann. Rev. Ecol. Syst.*, **18**, 23-42.

Hsiao T. H. (1993). – Molecular techniques for studying systematics and phylogeny of Chrysomelidae. In: Jolivet P., Cox M.L., Petitpierre E. (eds.), *Novel aspects of the biology of Chrysomelidae*. Dordrecht: Kluwer Academic Publ., 237-248.

Kumar S., Tamura K. & Nei M. (1993). – MEGA: Molecular Evolutionary Genetic Analysis, version 1.01. The Pennsylvania State University, University Park, PA 16802.

Leinaas H. P. & Fjellberg A. (1985). – Habitat structure and life history strategies of two partly sympatric and closely related, lichen feeding collembolan species. *Oikos*, **44**, 448-458.

MacArthur R. H. & Levins R. (1967). – The limiting similariry, convergence, and divergence of coexisting species. *Am. Nat.*, **101**, 377-385.

Mantovani E., Albarello D., Babbucci D. & Tamburelli C. (1992). – Recent geodynamic evolution of the central Mediterranean region. University of Siena, 88 p.

Mayr E. (1942). Systematics and the origin of species. Columbia University Press, New York.

Müller O. F. (1776). – Zoologiae Danicae prodromus. Typis Hallageriis, Copenhagen, xxxii + 202 p.

Mullis K. B., Faloona F., Scharf S., Saiki R. K., Horn G. T. & Erlich H. A. (1986). – Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symposium on Quantitative Biology*, **51**, 263-273.

Murphy R. W., Sites J. W. Jr., Buth D. G. & Haufler C. H. (1990). – Proteins I: isozyme electrophoresis. In: Hillis D. M. and Moritz C. (eds.), *Molecular systematics*, Sunderland, U.S.A: Sinauer Ass., 45-126.

Nei M. (1978). – Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583-590.

Nevo, E., Beiles A. & Ben Shlomo R. (1984). – The evolutionary significance of genetic diversity: ecological, demographic and life history correlates. In: Mani G.S. (ed.), *Evolutionary dynamics of genetic diversity. Lecture notes on biomathematics*. Springer-Verlag, **53**, 13-213.

Piertney S. B. & Carvalho G. R. (1994). – Microgeographic genetic differentiation in the intertidal isopod *Jaera albifrons* Leach. I. Spatial distribution of allozyme variation. *Proc. R. Soc. Lond. B*, **256**, 195-201.

Poinsot-Balaguer N. (1972). – Étude systématique et écologique des collemboles Isotomidae de Provence. *Ann. Soc. ent. Fr. (N.S.)*, **8**, 669-691.

Poinsot-Balaguer N. & Ferard M. (1983). – Contribution à l'étude du groupe *Isotomurus palustris* (Müller) (Collembole, Isotomidae). Redescription de *Isotomurus*

balteatus (Reuter, 1876 nov. comb.). *Nouv. Rev. Ent.*, **XIII**, 2, 147-156.

Reuter O. M. (1876). – Catalogous praecursorius Poduridarum Fenniae. *Medd. Soc. Fauna Flora Fenn.*, **1**, 78-86.

Robinson M. H. (1969). – Defenses against visually hunting predators. *Evol. Biol.*, **3**, 225-259.

Rogers J. S. (1972). – Measures of genetic similarity and genetic distance. *Studies in genet. VII*, Univ. Texas Publ., **7213**, 145-153.

Saitou N. & Nei M. (1987). – The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406-425.

Sevenster J. G. & van Alphen J. J. M. (1993). – A life history trade-off in *Drosophila* species and community structure in variable environments. *J. Anim. Ecol.*, **62**, 720-736.

Simon C. & Archie J. (1985). – An empirical demonstration of the lability of heterozygosity estimates. *Evolution*, **39**, 463-467.

Simon C., Franke A. & Martin A. (1991). – The Polymerase Chain Reaction: DNA extraction and amplification. In: Hewitt G. M., Johnston A. W. B., Young J. P. W. (eds.), *Molecular techniques in taxonomy*. NATO ASI Series, Berlin Heidelberg: Springer-Verlag, 329-355.

Simon C., Frati F., Beckenbach A., Crespi B., Liu H. & Flook P. (1994). – Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved Polymerase Chain Reaction primers. *Ann. Entomol. Soc. Am.*, **87** (6), 651-701.

Stach J. (1947). – The Apterygotan fauna of Poland in relation to the world-fauna of this group of Insects. Family Isotomidae. *Pol. Acad. Sc. & Lett. Cracow*, 1-488.

Swofford D. L. & Berlocher S. H. (1987). – Inferring evolutionary trees from gene frequency data under the principle of maximum parsimony. *Syst. Zool.*, **36**, 293-325.

Swofford D. L. & Selander R. B. (1981). – BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J. Heredity*, **72**, 281-283.